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Note

ATP—hemoglobin purification by ATP—agarose affinity chromatography*

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The plasma half-life and oxygen transport capacity of hemoglobin have been shown to be dramatically improved by modification with 2-nor-2-formyl pyridoxal phosphate [1] and periodate oxidized ATP [2]. Recently, Benesch et al. [3] proposed the use of pyridoxal phosphate cross-linked hemoglobin as a blood substitute in organ preservation, due to its extremely low oxygen affinity (i.e. $P_{50} \approx 60$ mmHg) and its ability to deliver oxygen at low temperature. However, the synthesis of 2,4-nor-formyl pyridoxal phosphate is difficult and the yield of cross-linked hemoglobin is limited to ca. 60% [1]. Therefore, for general use as a blood substitute, the ATP-modified hemoglobin (ATP—hemoglobin) prepared by Greenburg and Maffuid [2] appears more attractive because of the availability and relatively low cost of ATP and the known reaction chemistry involved. However, these authors appear to have some difficulty in purifying the ATP-modified hemoglobin from the reaction mixture by cation-exchange chromatography, which lead to extensive oxidation of the modified hemoglobin. They reported met-hemoglobin levels of ca. 30%.

We have recently demonstrated that ATP—agarose can be used for the purification of stroma-free hemoglobin [4]. In the present communication, we show that ATP-modified and ATP-unmodified hemoglobin can be separated by affinity chromatography on ATP—agarose gels. This separation procedure gives ATP—hemoglobin conjugate in greater than 98% purity, based on the removal of unmodified hemoglobin. Furthermore, the purified ATP—hemoglobin is shown to have better oxygen-releasing properties, i.e. lower oxygen affinity, than that of the reaction mixture and the unmodified hemoglobin.

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EXPERIMENTAL

Outdated human red blood cells were obtained from the Canadian Red Cross. Stroma-free hemoglobin (SFH) was prepared as described [4]. ATP and sodium metaperiodate were purchased from Sigma (St. Louis, MO, U.S.A.). Periodate-oxidized ATP was prepared according to the method of Lowe et al. [5]. ATP—agarose was prepared as described [6].

ATP—hemoglobin was prepared according to a modified method of Greenburg and Maffuid [2]. In the modified procedure, 2 ml of caprylic alcohol were added per 100 ml of 7% hemoglobin solution in 5 m*M* phosphate buffer (pH 7.0). Nitrogen was then bubbled for 2 h to deoxygenate the hemoglobin solution. The required amount of periodate-oxidized ATP in 50 m*M* Bis—Tris buffer (pH 7.0), which had been deoxygenated under nitrogen, was then added into the deoxygenated hemoglobin solution to give a molar ratio of 1.2:1 hemoglobin. Nitrogen was bubbled through the reaction mixture (ATP—hemoglobin solution) for another 90 min. The Schiff base of ATP—hemoglobin conjugate was reduced with sodium borohydride in 1 m*M* sodium hydroxide at a molar ratio of 20:1 sodium borohydride:hemoglobin. Again, nitrogen was bubbled through the mixture for another 2 h.

Upon completion of the reduction step, the solution was oxygenated and dialysed against 50 m*M* Bis—Tris (pH 7.0). Aliquots of the solution containing approximately 50 mg of hemoglobin were then passed through a column packed with 5 ml of ATP—agarose gel (ca. 30% of column capacity), which had been equilibrated with 50 m*M* Bis—Tris (pH 7.0). The unretained hemoglobin fraction was eluted by 5 vols. of the same buffer, and the retained fraction was then eluted by 0.2 *M* sodium chloride in 50 m*M* Bis—Tris (pH 7.0). The fractions were collected, concentrated and then dialysed against 50 m*M* Bis—Tris (pH 7.0) using a membrane concentrator (P-Micro ProDicon, Pierce, Rockford, IL, U.S.A.). The oxygen dissociation curve of each fraction was determined by an Aminco Hem-O-Scan oxygen dissociation analyser (American Instrument Company, Silver Spring, MD, U.S.A.). The P_{50} was read directly from the oxygen dissociation curve.

Samples not exceeding 100 μg of hemoglobin in 0.01 *M* malonate, pH 5.7 (buffer A), were applied to an HR 5/5 MonoS prepacked column (Pharmacia) equilibrated with buffer A. The fractions were eluted with a linear gradient of buffer B (0.3 *M* lithium chloride in buffer A) [7] using a Pharmacia LCC 500 liquid chromatographic system (Pharmacia, Montreal, Canada).

RESULTS AND DISCUSSION

ATP—agarose affinity chromatography

Fig. 1 shows the elution profile of the reaction mixture of ATP-modified hemoglobin on an analytical glass capillary column (1.8 cm × 1 mm) packed with 10 μl of ATP—agarose affinity gel. Unmodified hemoglobin, with its polyanion binding site intact, is capable of binding to the ATP—agarose and is therefore retained on the column while the modified hemoglobin, with one or more ATP molecule(s) covalently occupying its anion-binding site, is eluted as the unretained fraction. The unmodified hemoglobin can readily be recovered for further modification as described [4].

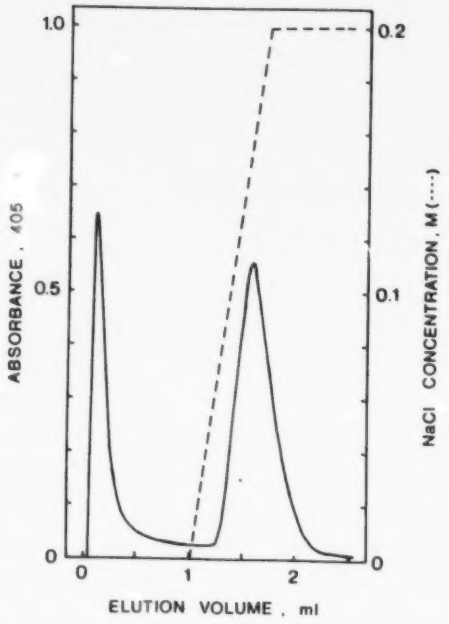

Fig. 1. Elution profile of the reaction mixture of periodate-oxidized ATP-modified hemoglobin (ATP—Hb) on an ATP—agarose affinity column. Buffer A is 50 m*M* Bis—Tris (pH 7.0) and buffer B is 0.2 *M* sodium chloride in buffer A. The flow-rate was 0.1 ml/min.

HPLC characterization of hemoglobin

Hemoglobin variants can be quantified by high-performance liquid chromatography (HPLC) using a MonoS analytical column (Pharmacia). Fig. 2A shows the elution profile of the unretained fraction compared with that of the reaction mixture. ATP—hemoglobin is eluted at a lower salt concentration than unmodified hemoglobin. Integration of the peak areas indicates that contamination of the unretained peak (ATP—Hb) by unmodified hemoglobin is $< 2\%$. On the other hand, the elution profile of the retained fraction is characteristic of unmodified hemoglobin (Fig. 2B). These results suggest near quantitative separation of the modified and unmodified hemoglobin from the reaction mixture by ATP—agarose affinity chromatography.

Oxygen-binding affinity of the hemoglobin solutions

The oxygen-binding affinities of the reaction mixture and the ATP—agarose retained and unretained fractions are shown in Fig. 3. The reaction mixture shows an oxygen dissociation curve intermediate between those of the retained and unretained fractions, consistent with the presence of modified and unmodified hemoglobin. The populations separated by ATP—agarose affinity chromatography, however, show distinct oxygen dissociation curves. The unretained fraction has a P_{50} of 35 mmHg, and the retained fraction a P_{50} of 6 mmHg.

Reaction mixtures of ATP-modified hemoglobin can therefore be effectively

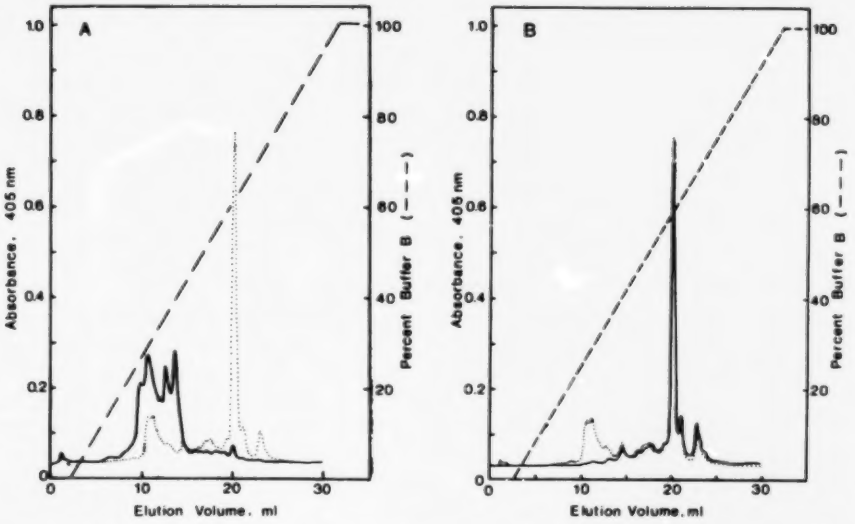

Fig. 2. Chromatogram of (A) 100 μg of unretained ATP—Hb fraction and (B) 100 μg of retained Hb fraction (after ATP—agarose column). The dotted chromatogram represents the reaction mixture before being passed through the ATP—agarose gel column. The column used was a Pharmacia HR 5/5 MonoS prepacked analytical column. Buffer A is 0.01 *M* malonate (pH 5.7) and buffer B is 0.3 *M* lithium chloride in buffer A. The flow-rate was 0.5 ml/min.

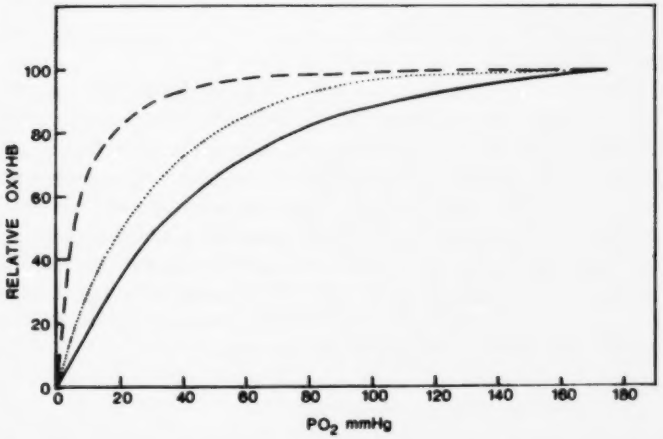

Fig. 3. Oxygen dissociation curve of the ATP—Hb reaction mixture (dotted line), ATP—agarose gel unretained fraction (solid line) and retained fraction (broken line) in 50 m*M* Bis—Tris (pH 7.0) at 37° C. Hemoglobin concentration is 3.5 g/dl.

separated by ATP—agarose affinity chromatography. The non-retention of ATP-modified hemoglobin on this column is attributed to the occupation of the polyanion binding site on hemoglobin by ATP, which inhibits its binding to the ATP immobilized in the gel. However, the exact site of attachment of ATP

to hemoglobin remains to be shown. Further proof that the unretained fraction is ATP—hemoglobin comes from its oxygen-binding affinity. In the experimental conditions used here, this fraction shows a P_{50} of 35 mmHg, identical to those of ATP—hemoglobin and hemoglobin—ATP—agarose complexes [4]. We have, therefore, demonstrated that affinity chromatography on ATP—agarose can purify an ATP—hemoglobin conjugate with physiological oxygen-binding affinity (P_{50} = 35 mmHg) and high (> 98%) purity.

Greenburg and Maffuid [2] have indicated that the plasma half-life of ATP—hemoglobin is ca. 14 h in the dog, as compared with just over 2 h for pyridoxylated hemoglobin. They have further suggested, without showing experimental data, that modification by ATP involves intramolecular cross-linking of hemoglobin, which stabilizes its native tetrameric form and prevents its rapid removal from the circulation [2]. It will be interesting to repeat such in vivo studies using ATP—hemoglobin purified as described above.

It is possible that polymerization of ATP—hemoglobin using glutaraldehyde will further improve its intravascular retention, similar to that of polymerized pyridoxylated hemoglobin [8]. Poly-ATP—hemoglobin can in principle attain concentrations of 14 g/dl, the normal concentration of hemoglobin in whole blood, without the excessive oncotic pressure associated with concentrated solutions of hemoglobin monomers. Furthermore, the 15—20% of ATP—hemoglobin, like pyridoxylated hemoglobin [8], may remain as monomers following polymerization but it should perform effectively as an oxygen carrier without posing the problems of rapid excretion since ATP—hemoglobin monomers have plasma half-lives of 12- 14 h in dogs [2]. The increased stability and purity of ATP—hemoglobin may also result in a reduction of the vasoconstrictor activity, which is a problem of hemoglobin solutions in vitro [9]. Another distinct advantage of the affinity chromatography purification scheme described above is that met-hemoglobin levels in the unretained fraction are less than 2%, as compared with levels of 30% reported by Greenburg and Maffuid [2]. Finally, the recovery of unmodified hemoglobin from the column allows it to be recycled through the modification procedure, thus potentially increasing the overall yield of ATP—hemoglobin.

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